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The Dietary Supplement Protandim® Decreases Plasma Osteopontin and Improves Markers of Oxidative Stress in Muscular Dystrophy *Mdx* Mice

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Abstract

Therapeutic options for Duchenne muscular dystrophy (DMD), the most common and lethal neuromuscular disorder in children, remain elusive. Oxidative damage is implicated as a pertinent factor involved in its pathogenesis. Protandim® is an over-the-counter supplement with the ability

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DISCLOSURE

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to induce antioxidant enzymes. In this study we investigated whether Protandim® provided benefit using surrogate markers and functional measures in the dystrophin-deficient (*mdx*)mouse model of DMD. Male 3-week-old *mdx* mice were randomized into two treatment groups: control (receiving standard rodent chow) and Protandim®-supplemented standard rodent chow. The diets were continued for 6-week and 6-month studies. The endpoints included the oxidative stress marker thiobarbituric acid-reactive substances (TBARS), plasma osteopontin (OPN), plasma paraoxonase (PON1) activity, H&E histology, gadolinium-enhanced magnetic resonance imaging (MRI) of leg muscle and motor functional measurements. The Protandim® chow diet in *mdx* mice for 6 months was safe and well tolerated. After 6 months of Protandim®, a 48% average decrease in plasma TBARS was seen; 0.92 nmol/mg protein in controls versus 0.48 nmol/mg protein in the Protandim® group ($p = .006$). At 6 months, plasma OPN was decreased by 57% ($p = .001$) in the Protandim®-treated mice. Protandim® increased the plasma antioxidant enzyme PON1 activity by 35% ($p = .018$). After 6 months, the *mdx* mice with Protandim® showed 38% less MRI signal abnormality ($p = .07$) than mice on control diet. In this 6-month *mdx* mouse study, Protandim® did not significantly alter motor function nor histological criteria.

Keywords

Duchenne muscular dystrophy; protandim®; *mdx* mice; dystrophin; dystrophic muscle; paraoxonase; osteopontin; oxidative stress

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder that occurs in 1 in 3,500 live male births and is the most common fatal genetic disorder in children (Emery, 1991). It is caused by loss-of-function mutations in the gene dystrophin that encodes a massive muscle sub-sarcolemmal cytoskeletal protein. The pathogenesis of DMD is frequently studied in the dystrophic *mdx* mouse model (Bulfield, Siller, Wight, & Moore, 1984; Sicinski et al., 1989). However, despite the complete deficiency of dystrophin protein, *mdx* mice have a near-normal life span. At approximately 3–6 weeks of postnatal age, *mdx* mice develop a crisis phase of muscle necrosis followed by regeneration (McArdle et al., 1998). The mice are often studied only during the first 6 weeks of their lives before a stable regenerative phase ensues that achieves a mild clinical adult mouse phenotype.

There is no cure for DMD and the only medications proven to favorably alter its natural history are corticosteroids (Mendell et al., 1989). There is evidence from randomized controlled trials that glucocorticoid therapy in DMD improves muscle strength and function in the short-term period of 6 months to 2 years (Manzur, Kuntzer, Pike, & Swan, 2004). However, the long-term efficacy of corticosteroids in DMD with randomized placebo-controlled trials will likely never be studied given its orphan disease state and obvious side effects. There are a number of chronic corticosteroid regimens (daily versus intermittent) and most recommended dose appears to be prednisone 0.75 mg/kg/day or deflazacort at 0.9 mg/kg/day. The use of corticosteroids is associated with numerous side effects particularly weight gain, stunted height/growth, cataracts, osteoporosis, hypertension, diabetes, hirsutism, and mood/behavioral changes (Kelly et al., 2008; Manzur, Kuntzer, Pike, & Swan, 2004; Wong & Christopher, 2002). Thus, better treatments to augment or supplant corticosteroid use in DMD would be of immense value. To find a cocktail of other compounds that could lower the dose needed of corticosteroid would be of tremendous clinical value to attenuate the chronic corticosteroid side-effect burden.

Oxidative stress is a significant pathologic factor in DMD. Skeletal muscles of *mdx* mice demonstrate increased quantities of oxidative damage markers including the by-products of lipid peroxidation and carbonyls (Haycock, Mac, & Mantle, 1998; Ragusa, Chow, & Porter,

1997). The dystrophin-deficient myotubes are highly susceptible to cellular injury, particularly loss of membrane integrity, when exposed to reactive oxygen species (Disatnik, Chamberlain, & Rando, 2000; Rando, Disatnik, Yu, & Franco, 1998). Several co-morbidities in DMD, including muscle fatigue and cardiomyopathy, are associated with increased oxidative stress (Bia et al., 1999; Chi et al., 1987; Mohr, Hallak, de Boitte, Lapetina, & Brune, 1999).

Protandim® (LifeVantage Corp., San Diego, CA) is a dietary supplement available as an over-the-counter herbal supplement (once daily capsule of 675 mg) (Nelson, Bose, Grunwald, Myhill, & McCord, 2006). It is composed of the following phtyochemicals: (1) *Bacopa monniera* extract (45% bacosides), 150 mg; (2) *Silybum marianum* extract (70%–80% silymarin), 225 mg; (3) *Withania somnifera* (Indian ginseng) powder, 150 mg; (4) green tea extract (*Camellia sinensis*, 98% polyphenols and 45% epigallocatechin-3 gallate), 75 mg; and (5) curcumin (95%) from *Curcuma longa*, 75 mg. Individual ingredients of Protandim® are well-known antioxidants that cause induction of SOD and catalase in rodents and diminish cellular lipid peroxidation (Joe, Vijaykumar, & Lokesh, 2004; Kishore & Singh, 2005; Lang, Deak, Muzes, Pronai, & Feher, 1993; Mandel, Weinreb, Amit, & Youdim, 2004; Rasool & Varalakshmi, 2007).

The effect of a single dose per day (675 mg) of Protandim®, for 30–120 days, has been tested on 29 healthy human volunteers ranging in age from 20 to 78 years (Nelson, Bose, Grunwald, Myhill, & McCord, 2006). Erythrocytes were assayed for superoxide dismutase (SOD) and catalase, and plasma was assayed for thiobarbituric acid-reacting substances (TBARS). Before supplementation, the levels of TBARS showed a strong age-dependent increase. After 30 days of supplementation, TBARS declined by an average of 40% and the age-dependent increase was eliminated. By 120 days, erythrocyte SOD increased by 30% and catalase by 54%.

The mechanism of action of Protandim® has been shown to be through activation of the transcription factor nuclear factor E2-related factor 2 (Nrf2) by a mechanism involving multiple signaling pathways and substantial synergy among the five active ingredients (Velmurugan, Alam, McCord, & Pugazhenthi, 2009). Nrf2 is known to induce many antioxidant enzymes via the antioxidant response element in their promoters, including enzymes involved in the synthesis and metabolism of glutathione. The synergy obtained in the composition enables activation of Nrf2 at very low concentrations of the individual active ingredients (Velmurugan, Alam, McCord, & Pugazhenthi, 2009). Protandim® provided substantial chemoprevention in a two-stage skin carcinogenesis study in the mouse. Protandim®-supplemented mice showed a 33% reduction in skin tumor incidence and a 57% reduction in tumor multiplicity (Liu et al., 2009). Protandim® has also been shown to induce the antioxidant transcription factor Nrf2 that prevents cardiac oxidative stress (Bogaard et al., 2009). Nrf2 preserves the expression of vascular endothelial growth factor and prevents right ventricular failure without modifying lung angioproliferation.

The by-products of free radical damage to polyunsaturated fatty acids react with thiobarbituric acid to form products that may be assayed as an index of oxidative damage and lipid peroxidation (Armstrong & Browne, 1994). The concentration of TBARS has been found to be significantly elevated in skeletal muscle of boys with DMD as well as *mdx* mice and is a reliable indicator of oxidative stress levels in dystrophic muscle (Faist, Koenig, Hoeger, & Elmadfa, 1998; Jackson, Jones, & Edwards, 1984; Kar & Pearson, 1979). In the present work, we evaluated the 6-week and 6-month use of Protandim® in *mdx* mice using a placebo-controlled design. The primary endpoint of the study was to assess the impact of Protandim®-enriched diet versus control diet on *mdx* mice plasma and skeletal muscle homogenate levels of TBARS. Secondary endpoints included a comparison of the plasma levels of profibrotic factor osteopontin (OPN, also known as SPP1) and antioxidant enzyme paraoxonase (PON1) as arylesterase activity, Hematoxylin and Eosin (H&E) muscle histology, region-of-interest

(ROI)-quantitative measurement of percentage gadolinium-enhanced muscle areas using magnetic resonance imagine (MRI) (Voisin et al., 2005) and functional measures including voluntary running (cumulative distances), time (min) to exhaustion running downhill and spontaneous cage activity (beam break counts).

MATERIALS AND METHODS

Animals, Specimen Collection, and Preparation

Adult *mdx* (C57BL/10ScSn-*mdx*) mice were originally obtained from Jackson Laboratory (JAXR®, BarHarbor, ME). All *mdx* mice were housed and handled in accordance with guidelines and procedures approved by the Institutional Animal Care and Use Committee. *Mdx* mice were kept at a research lab at the University of Colorado Health Sciences Center inside cages in a traffic-free, quiet, and dim environment. Male *mdx* mice with confirmatory PCR genotyped from tail DNA (data not shown) were utilized for these studies.

Treatment Protocol

Diets were provided ad lib to either adult *mdx* mice or breeder females. Protandim® was formulated into standard Harlan Teklad 2018S rodent chow (custom order through Research Diets, Inc., New Brunswick, NY) for the intervention group while the control group ate the same chow without Protandim®. The mice received Protandim® at a dosage of approximately 457 mg/m^2, calculated according to Reagan-Shawet al. (2008), which is nearly equivalent to the manufacturers recommended human dose of 675 mg per day for a 60 kg adult, or 422 mg/m^2. For the 6-week study, the treatment was given to breeder females so that the 6-week *mdx* mice group was exposed to Protandim® through placental absorption. After birth, the pups were kept on the Protandim® diet to 6 weeks of age when they were euthanized.

The chow was provided at 3–6 weeks of postnatal age during known muscle necrosis phase in *mdx* mouse. During this stage, the mice do not appear overtly crippled and are active, but their muscle tissues demonstrate most marked features of histopathology. In the second part of the study, Protandim® chow diet was provided for 6 months to *mdx* mice greater than 8 weeks of age where more histopathology was exacerbated by having *mdx* mice run downhill (5% grade) on treadmills which increases eccentric damage to muscles. The *mdx* mice at this age have stably regenerated muscles with minor markers of past regeneration events particularly central nuclei instead of peripheral muscle nuclei.

Blood Collection

Blood (50–100 μl) was collected via retro-orbital eye bleed of time of harvest. Isoflurane inhalant via a rodent anesthesia funnel mask was given to the mice for approximately 2 min prior to the retro-orbital eye bleed. Additional local anesthesia of preparacaine eyedrops was given before blood sample was collected. Each mouse was pinch-tested to verify adequate anesthesia. The entire procedure took less than 5 min. The mice woke up usually within 2–3 min after removal of isoflurane. Blood samples allowed serum creatine kinase (CK), plasma TBARS, OPN, and PON1 analyses.

Tissue Collection

After completion of the dietary period, mice were euthanized with a mixture containing ketamine and xylazine. Cervical dislocation was performed and then skeletal muscles, including gastrocnemius, tibialis anterior, rectus femoris and hamstring, from the one leg were dissected and harvested for histological studies using quick freezing technique. The contralateral leg muscles were harvested for biochemical and western blot assays.

Mdx mice were randomized into the two groups. Blood samples, tissue samples, and images were collected, coded, and subsequently analyzed in a blinded fashion to laboratory personnel so that dietary group was unknown until studies were completed then code revealed.

Thiobarbituric Acid-Reacting Substances Assay (TBARS)—Thiobarbituric acid-reacting substances were measured by a method described previously (Ohkawa, Ohishi, & Yagi, 1978, 1979). The reagents included thiobarbituric acid reagent (0.8% w/v), sodium dodecyl sulfate (SDS) reagent (8.1% w/v), acetic acid reagent (20% v/v), n-butanol/pyridine mixture (15:1, v/v), and malonaldehyde standard (100 nmol/ml).

The reaction mixture was comprised of 50 μl of 8.1% SDS, 0.375 ml of 20% acetic acid reagent, and 0.375 ml of 0.8% thiobarbituric acid. Distilled water was added so that the total sample from plasma or muscle homogenate/water volume became 200 μl and the total reaction volume 1.0 ml. This mixture was heated in boiling water for 60 min and then cooled under tap water. Distilled water (0.250 ml) and 1.25 ml of n-butanol/pyridine mixture were added to the mixture. Then, the mixture was shaken vigorously and centrifuged at 500–1000 g for 10 min. The amount of color formation at an absorbance wavelength of 532 nm (A532) was measured against a reaction mixture blank. The A532 was plotted against the malonaldehyde standard solution (nmol) to determine the plasma TBARS level.

Assessment of Plasma Osteopontin (OPN)—Plasma samples from *mdx* mice maintained for 6 months on control ($n = 11$) or Protandim®-supplemented ($n = 15$) diets were subjected to Western blot analysis for OPN. Plasma samples (1 μl) were chromatographed on 4%–20% SDS-PAGE (BioRad, Hercules, CA). Blots were probed with anti-OPN (aka SPP1) (mouse monoclonal anti-SPP1 antibody diluted 1:2,000, Millipore, Billerica, MA). Two bands were visualized: band 1 at 50 kDa and band 2 at 25 kDa. The bands were scanned and digitally integrated using a Kodak Image Station 440CF and 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

Plasma Paraoxonase (PON1) Activity—PON1 arylesterase activity was measured in plasma from *mdx* mice at 6 weeks of age and at 6 months, on control diet and Protandim-supplemented diet. Arylesterase activity was measured spectrophotometrically as described by Eckerson et al. (1983) using phenylacetate (Sigma, St. Louis, MO) as substrate. The reaction mixture contained 1 mM phenylacetate, 9 mM of Tris/HCL, and 0.9 mM of $CaCl_2$ at pH 8.0. The increase in absorbance at 270 nm was read using a molar extinction coefficient of 1,310 M^{-1} cm^{-1}. Arylesterase activity is expressed in U ml^{-1} plasma.

Assessment of Disease Progression

Histology—Areas of degeneration or regeneration (DRG) were measured and compared with the total area of the examined muscle using H&E staining. The H&E staining is used to detect abnormal variation in fiber size, degenerating and regenerating fibers, immune cell infiltration, and increased fibrosis in *mdx* muscles. Control mice muscles do not have these pathologic features (data not shown).

Gadolinium-Enhanced MRI of Skeletal Muscle—Gadolinium-enhanced MRI images of the gastrocnemius and rectus muscles of the anesthetized *mdx* mice were obtained. A custom home-built nonmagnetic mouse holder was used to keep each mouse stabilized with good airway protection while anesthetized for imaging. Imaging was performed in 4.7 Tesla Bruker MRI at the University of Colorado Cancer Center Core Facility Bioimaging Suite. Region-of-Interest ROI-quantitative measurements of percentage gadolinium-enhanced areas, representing excessive muscle cell permeability, in the Protandim® and control diet groups were obtained. Scar perfusion and vascularity was obtained using dynamic contrast-enhanced

MRI using gadolinium-based contrast agent as a diffusible extracellular tracer. The procedure included an injection of gadolinium-DTPA bismethylamide (gadodiamide, OMNISCANR®) into the tail vein of the *mdx* mice. OMNISCANR® (Amersham Health) is an FDA approved injectable, nonionic MRI contrast agent which is broadly used in human clinical MRI. Control muscles do not have gadolinium-enhancing lesions (data not shown).

Measures of Muscle Performance—Voluntary exercise performance was assessed on mouse running wheels (Hara et al., 2002). The *mdx* mice were housed with 4.5 inch running wheels (Super Pet Mini Run-Around) adapted with Sigma Bicycle odometers (Sigma Sport BC 401) that records speeds and cumulative distances run. Weekly running distances (km) were recorded over the treatment period.

Forced 5% downhill treadmill running was performed on motorized treadmill. The treadmill had a 45-degree slope at 10 m/min. Mice ran 7.5 min for 7.5 m/min pace, then 7.5 additional min at 10 m/min pace. This protocol results in eccentric muscle damage to exacerbate the *mdx* mouse skeletal muscle phenotype. With treadmill run to exhaustion at speeds of up to 10 m/min, all mice eventually stop and the time to exhaustion (minutes) is recorded.

Motion beam detectors: To quantify spontaneous locomotor activity, experimental mice and control littermates were placed in individual automated photocell activity cages (29 × 50 cm) with twelve 2 cm high infrared beam detectors in a 4 × 8 grid (San Diego Instruments, San Diego, CA). *Mdx* mice were habituated and recordings were then made (i) during their active nocturnal dark 12-hour cycle for overnight baseline activity, and (ii) during their normal sleep cycle for post-downhill run recovery.

Statistics

On the basis of the effect size projected from other published mouse data, we anticipated a size of effect "variance" of 25%. Given this size of effect, our preliminary power analysis (alpha < .05) required at least 9.4 *mdx* mice per group and time point. In some of our experiments we had multiple time points with some attempts to minimize number of *mdx* mice needed by doing nonterminal studies such as imaging with the MRI. However, for satisfactory statistics, when the sizes of effects (variance) were modest we aimed for a larger sample size of up to 10 mice per group to raise our power of analysis.

Group or pairwise parametric or nonparametric comparisons were done using NCSS software (NCSS, Kaysville, UT, USA). A p-value of <.05 was considered significant. Average plasma TBARS, muscle TBARS, plasma PON1, plasma OPN, and running distances were determined for each *mdx* mouse. These values were averaged for Protandim® and control mice for an overall value. Statistical differences between means were analyzed using Student's t-test.

RESULTS

Morbidity and mortality comparisons in *mdx* mice supplemented with Protandim® or control rodent diets are summarized in Table 1. The average body weight between the Protandim® (n = 13) and control (n = 12) rodent diet groups increased (at equivalent rates) during the 22-day period, in which body weight and health were monitored. There were no observations of adverse events or growth impairments in the Protandim®-treated mice. In fact, one interesting incidental observation was the glossier sheen of fur seen in all *mdx* mice on 6 months of Protandim diet (not shown). This incidental finding did make our blinding efforts of the two groups important to ensure sample identifiers were coded and then blinded to subsequent analysis. Protandim® treated mice demonstrated no difference in serum CK (data not shown).

After 6 months of taking Protandim®, a statistically significant 48% average decrease in plasma TBARS was seen (Figure 1); 0.48 nmol/mg in Protandim® group (n = 12; p = .006) versus 0.92 nmol/mg in controls (n = 9). A comparison of hamstring muscle TBARS (Figures 2(a) and (b)) in the two groups after 6 weeks and 6 months was not significant. The average muscle TBARS after 6 weeks was lower in 11 young *mdx* mice fed with Protandim® (8.8 nmol/mg ± 3.1 SD) compared with 11 control diet *mdx* mice (11.4 nmol/mg ± 5.9 SD). Similarly, the average muscle TBARS after 6 months was lower in 16 adult *mdx* mice fed with Protandim® (18.4 nmol/mg ± 10.1 SD) compared with nine adult *mdx* mice on control diet (30.1 nmol/mg ± 21.3 SD).

Western blot analysis for OPN in plasma samples from *mdx* mice maintained for 6 months on control (n = 11) or Protandim®-supplemented (n = 15) diets revealed two bands: band 1 at 50 kDa and band 2 at 25 kDa. The bands were scanned and digitally integrated, and the results are shown in Figure 3. Both bands were reduced in intensity by Protandim® supplementation and to approximately the same extent (band 1 by 53%, p = .007; band 2 by 60%, p = .004; the sum of the two bands by 57%, p = .001).

Measurement of plasma paraoxonase (PON1) arylesterase activity at 6 weeks showed that Protandim®-supplemented animals (n=9) averaged 30.1±2.6 U/ml, or 35% more than control animals (22.3 ± 3.1 U/ml, n = 6). Similarly, at 6 months Protandim®-supplemented animals (n = 15) averaged 11.7 ± 1.4 U/ml, or 36% more than control animals (8.6 ± 1.1 U/ml, n = 11), even though plasma PON1 activity dropped for both groups as the animals aged from 6 weeks to 6 months. When the control and supplemented animals were normalized to the mean of the control group for their respective ages, the pooled data showed that Protandim® supplementation (n = 24) increased PON1 activity by 35% (p = .018) over controls (n = 17), and the results are seen in Figure 4.

The H&E comparisons of the gastrocnemius and rectus muscles in *mdx* mice fed with Protandim® or control diet both exhibited equivalent features of abnormal variation in fiber size, degenerating and regenerating fibers, immune cell infiltration, and fibrosis, thus did not demonstrate a significant difference at 6 weeks (Figure 5) or 6 months (Figures 6(a) and (b)).

A comparison of ROI-quantitative measurements of percentage gadolinium-enhanced scar areas linked to abnormal membrane permeability in the *mdx* mice at 6 months showed 38% less signal in the Protandim® group (15.6% ± 6.2% SD, n = 15) than in the control group (25.06% ± 13.6% SD, n = 10), but this difference did not quite reach statistical significance (p = .07) (see Figure 7). In Figure 8, representative cross-sectional lower limb MRI images are shown from 2 control and 2 Protandim® diet mdx mice. The control group exhibited a greater ROI percentage damage on the MRI images than the Protandim® group, as indicated by increased areas of abnormal gad-signal enhancement. Muscle images of wild-type mice (n = 6) even if run were found to have no ROI percentage damage features (data not shown).

No significant differences were observed when comparing motor function between *mdx* mice fed with Protandim® or control diets after 6 weeks and 6 months. No differences were found in voluntary running wheel distances after 6 weeks (p = .06; Protandim® n = 9, control n = 12) or 6 months of treatment (p = 0.6; Protandim® n = 8, control n = 6) between Protandim® and control diet-fed *mdx* mice. Time to downhill running exhaustion was not statistically different (data not shown). Baseline cage activity was recorded using the motion sensor detectors over a two-night period and were also not statistically different.

DISCUSSION

Major findings of this study were significant reductions of plasma TBARS and the profibrotic factor osteopontin, and a significant increase in plasma PON1 activity in *mdx* mice after the

administration of the dietary supplement Protandim® for 6 months at a dosage equivalent to that recommended for humans. TBARS is one of the most widely used markers of lipid peroxidation in published literature (Walter et al., 2004). Increased levels have been demonstrated in animal models of muscular dystrophy (Faist, Koenig, Hoeger, & Elmadfa, 1998; Mizuno, 1984; Ohta & Mizuno, 1984) and humans (Kar & Pearson, 1979). A significant increase in the plasma activity of the antioxidant enzyme paraoxonase (PON1) further supported the decreased oxidative stress in Protandim®-supplemented animals. PON1 has been shown to be inactivated by oxidative stress (Nguyen & Sok, 2003). PON1 protects LDL against oxidative modification, and low PON1 activity is strongly associated with increased risk for cardiovascular disease (Soran, Younis, Charlton-Menys, & Durrington, 2009). These three independent findings are consistent with a favorable response from Protandim diet in the face of oxidative stress in the *mdx* mouse.

The increased action of oxidative stress in DMD is indicated by several factors including increased excretion of 8-hydroxy-2′-deoxyguanosine (Rodriguez & Tarnopolsky, 2003), increased lipid peroxidation products such as plasma TBARS (Haycock, Mac, & Mantle, 1998; Hunter & Mohamed, 1986), significant disturbances of metabolic pathways that supply the reactions necessary for efficient glutathione regeneration in dystrophic muscle (Dudley et al., 2006), increased sensitivity of dystrophin-deficient cells to injury from oxidative stress (Donatella Degl'Innocenti APFRGR, 1999), and lipofuscin accumulation in dystrophic muscle (Nakae et al., 2004). It has been demonstrated that the dystrophin protein complex may have important regulatory or signaling properties in terms of cell survival and antioxidant defense mechanisms (Disatnik, Chamberlain, & Rando, 2000).

More recently, it has been shown that skeletal muscles of DMD patients and *mdx* mice experience recurrent bouts of functional ischemia during muscle contraction because of a loss of neuronal nitric oxide synthase (nNOS) from subsarcolemma (Chang et al., 1996; Dudley et al., 2006; Sander et al., 2000). As a consequence, the production of nitric oxide (NO) is tremendously reduced in dystrophin-deficient muscles (Wehling, Spencer, & Tidball, 2001). Because NO is a reactant with other free radicals, the loss of its production in dystrophic muscle changes the redox environment in muscle increasing oxidative stress (Stamler, Singel, & Loscalzo, 1992). Furthermore, skeletal muscle-derived NO plays a key role in the regulation of blood flow within exercising skeletal muscle by blunting the vasoconstrictor response to α-adrenergic receptor activation. The loss of nNOS in the *mdx* mice causes a defect in the normal ability of skeletal muscle contraction to attenuate α-adrenergic vasoconstriction (Thomas et al., 1998), leading to ischemic-perfusion defects. Reperfusion after ischemia is a well-established cause of oxidative stress and particularly relevant for pathogenesis of dystrophic deficiency in DMD (Mendell, Engel, & Derrer, 1971). Dudley and colleagues (2006) studied the status of critical metabolic pathways of the glutathione system in the skeletal muscle of *mdx* mice and characterized the dynamic in vivo responses of the glutathione system to an acute challenge by ischemia-reperfusion. Their data suggest that dystrophin-deficient muscles adapt to chronic ongoing oxidative stress by upregulating glutathione-associated antioxidant enzymes and that this affords a degree of protection against acute bouts of oxidative stress induced by ischemic dystrophic muscle in *mdx* mice. In cultured human brain-derived cells, Protandim® was shown to increase glutathione concentration by two- to fourfold (Velmurugan, Alam, McCord, & Pugazhenthi, 2009). Increased production of glutathione might be particularly desirable in DMD.

In this study, we demonstrate that the Protandim® chow in *mdx* mice significantly reduces plasma TBARS in *mdx* mice after 6 months. The nutritional supplement Protandim® has been shown to reduce plasma TBARS by approximately 40% in healthy human subjects after 4 months of treatment, with significant inductions of the antioxidant enzymes superoxide dismutase and catalase (Nelson, Bose, Grunwald, Myhill, & McCord, 2006). Our data

demonstrate a remarkably similar finding in *mdx* mice with a reduction in plasma TBARS of approximately 48% after 6 months of treatment. Our plasma TBARS result suggests that induction of antioxidant enzymes by a combination of phytochemicals reduces oxidative stress in *mdx* mice.

Osteopontin (OPN) is a pleiotropic protein first described in the context of cellular transformation (Senger, Wirth, & Hynes, 1979). The protein was renamed secreted phosphoprotein-1 (SPP1) to avoid the implication of a single specific function, but it is still largely referred to as osteopontin. Recent reviews have summarized its roles in mineralization of tissues (Gerstenfeld, 1999), in regulating chronic inflammation and vascular diseases (Scatena, Liaw, & Giachelli, 2007), in the immune system (Gravallese, 2003), in cardiovascular disease (Okamoto, 2007), and in cancer (Wu et al., 2007).

Clinically, OPN plasma levels are elevated in many diseases characterized by chronic inflammation or fibrosis. Recent studies in rats (Kramer, Sandner, Klein, & Krahn, 2008) and humans (Rosenberg et al., 2008) have validated plasma OPN as a biomarker of chronic heart failure and as an independent predictor of death. OPN has a profibrotic effect in animal models of lung fibrosis and is the most upregulated gene (20-fold) in human idiopathic pulmonary fibrosis (IPF; Pardo et al., 2005). OPN is a potential target for therapeutic intervention in DMD, a life-limiting pediatric genetic disease.

In two studies of gene expression in the *mdx* mouse, it was noted that the OPN (SPP1) gene was also the most upregulated, documented at both the message and protein levels (Porter et al., 2002; Turk et al., 2006). This suggests that the overexpression of OPN may also be a contributing factor in the relentless clinical progression of DMD, as proposed by Pardo et al. (2005) for idiopathic pulmonary fibrosis.

A recent study by Vetrone et al. (2009) found that OPN promotes fibrosis in *mdx* mouse muscle by modulating immune cell subsets and intramuscular TGF-β. The genetic elimination of OPN expression in the *mdx* mouse correlated with increased strength and reduced diaphragm and cardiac fibrosis, suggesting that OPN may be a promising therapeutic target for reducing inflammation and fibrosis in individuals with DMD.

While the Protandim®-activated transcription factor Nrf2 upregulates the expression of many antioxidant enzymes, a smaller number of proinflammatory genes are down-regulated by Nrf2 (Chen, Dodd, & Thomas, 2006), including the inducible nitric oxide synthase (iNOS or nos2) and matrix metalloproteinase-9 (Kim et al., 2007). Stable overexpression of Nrf2 significantly decreases mRNA expression of OPN (Hinoi et al., 2007), suggesting that it too is negatively regulated by Nrf2. Our result that Protandim® supplementation of *mdx* mice decreased plasma OPN by 57% is consistent with these observations.

The ability to pharmacologically adjust OPN levels may be very useful therapeutically. In a wound healing/inflammation model, the use of antisense oligodeoxynucleotides (siRNA) to knockdown OPN expression by about 50% led to rapid repair and reduced fibrosis and scarring (Mori, Shaw, & Martin, 2008). Similar results were seen in a mouse model of fulminant hepatitis where treatment with OPN siRNA led to a significant reduction in liver injury (Saito et al., 2007). In the present study, an approximate 50% reduction in plasma OPN levels was achieved in mice by supplementation with Protandim®. Thus, OPN may be an important drug target for the clinical management of diseases involving chronic inflammation and fibrosis, including DMD and related muscular dystrophies. Perhaps noteworthy is the fact that glucocorticoids represent the only widely used therapy for DMD, and dexamethasone has been shown to induce the OPN gene by up to 20-fold (Pockwinse, JL, Lian, Stein, 1995).

An interesting recent development in the story of OPN is the recognition that the protein is a substrate for tissue transglutaminase-2 (TG2) that can introduce covalent cross-links between subunits of OPN (Kaartinen, Pirhonen, Linnala-Kankkunen, & Maenpaa, 1999). TG2 release by injured arteries appears to be a necessary step for arterial calcification (Johnson, Polewski, & Terkeltaub, 2008). Remarkably, many of the actions of OPN were dramatically enhanced when cells were treated with TG2-polymerized OPN (Higashikawa, Eboshida, & Yokosaki, 2007). Much of the explanation may reside in the fact that polymerized OPN binds more tightly to collagen fibrils in the extracellular matrix (Kaartinen, Pirhonen, Linnala-Kankkunen, & Maenpaa, 1999). These enhanced functions imply that while increased plasma concentrations of OPN have been correlated with many disease states as summarized above, the polymerization state of the protein might be an even more important factor in the pathophysiological functions of OPN. In this regard, it is noteworthy that we observed both 25 kDa and 50 kDa forms of OPN in *mdx* mouse plasma (see Figure 8), and both forms were reduced >50% by Protandim®. [We have also observed a high molecular weight (120 kDa) form of plasma OPN in boys with DMD which is apparently a tetramer of the normal 31 kDa plasma OPN. Protandim® supplementation of these boys decreased this polymerized OPN by an average of 44% (P. Vondracek and J. M. McCord, unpublished data).]

We found that the plasma activity of PON1 was significantly higher in *mdx* mice supplemented with Protandim®, whether for 6 weeks or 6 months. PON1 is susceptible to being partially inactivated by the metabolic products of oxidative stress—specifically, by oxidized glutathione (GSSG) through the process of S-glutathionylation and by products of lipid peroxidation, which can also react with the free sulfhydryl group of the enzyme (Aviram et al., 1999; Rozenberg & Aviram, 2006). The prevention of these processes in Protandim®-supplemented mice presumably accounts for most if not all of the increased PON1 activity. The reversal, or partial reversal, of these posttranslational modifications is brought about by incubation of the modified protein with 10 mM dithiothreitol. Analysis reveals that after 6 weeks of supplementation with Protandim®, the amount of S-glutathionylated or aldehyde-adducted PON1 is reduced by 54%, suggesting that both GSSG concentrations and lipid peroxidation products may be decreased significantly by Protandim®.

There is evidence that shows individual components within Protandim® can impact DMD by reducing oxidative damage and lipid peroxidation. The use of green tea extract and curcumin is worth mentioning. One study demonstrated that *mdx* mice that began voluntary wheel running at the age of 21 days demonstrated that running distance was increased by approximately 128% by the use of 0.5% green tea extract in the diet (Call et al., 2008). Green tea extract has been demonstrated to reduce muscle necrosis in *mdx* mice by antioxidant mechanisms (Buetler, Renard, Offord, Schneider, & Ruegg, 2002; Dorchies et al., 2006; Nakae et al., 2008). Curcumin has shown to enhance speed recovery of both voluntary (wheel running) and involuntary (treadmill) running performance following exercise-induced muscle damage (Davis et al., 2007). These performance effects were reflected by a reduction in plasma creatine kinase and inflammatory cytokine concentrations in muscle.

Our study has several limitations. First, the relatively short 6-month duration of the study may be one reason why a large impact of Protandim® was not observed with statistical significant differences in muscle TBARs, muscle H&E histology, MRI muscle imaging, nor muscle function measurements. Second, in the morphometric analyses of muscles in the treated versus untreated *mdx* mice, the degeneration–regeneration index can be affected by artifacts and there is possibility of observer bias. The role of TBARS as a surrogate marker for oxidative stress in DMD is implicated but has not been fully validated as a biomarker to reflect a direct correlate to the clinical function of an *mdx* mouse, much less a boy with DMD. There is also uncertainty of the significance of plasma TBARS versus tissues TBARS in the pathogenesis of DMD. Some of the long-term impacts of reduced oxidative damage may have been more detectable

with other outcome measures including cardiac, respiratory function, grip strength-testing, and total lifespan, which were not defined in this study. Finally, the *mdx* mouse may not be the most affected model to test a potential DMD interventional agent such as Protandim, which appears to exert effects through antioxidant pathways.

Despite these limitations, the study provides evidence of reduced oxidative damage, and perhaps of a reduced profibrotic state, with the use of Protandim® in *mdx* mice. The use of Protandim® did not cause any untoward side effects and overall the compound appeared safe. Pharmacokinetic studies and long-term trials of Protandim® in *mdx* mice and humans with DMD are required to determine its impact on DMD disease progression and survival.

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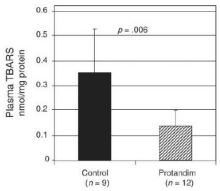

FIGURE 1.

Plasma TBARS in 6 months Protandim® versus control rodent diet fed mdx mice show a 48% decrease; 0.92 nmol/mg in controls ($n = 9$) versus 0.48 nmol/mg in Protandim® group ($n = 12$; $p = .006$).

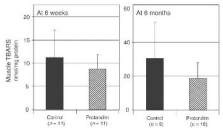

FIGURE 2.

Muscle TBARS analysis of Protandim® versus control diet fed mdx mice demonstrates that (a) average muscle TBARS after 6 weeks was lower if fed Protandim® (*n* = 11) (8.8 nmol/mg ± 3.1 SD) compared with control diet (*n* = 11) (11.4 nmol/mg ± 5.9 SD) (b) average muscle TBARS after 6 months was lower in mdx mice fed with Protandim® (*n* = 16) (18.4 nmol/mg ± 10.1 SD) compared with control diet (*n* = 9) (30.1 nmol/mg ± 21.3 SD).

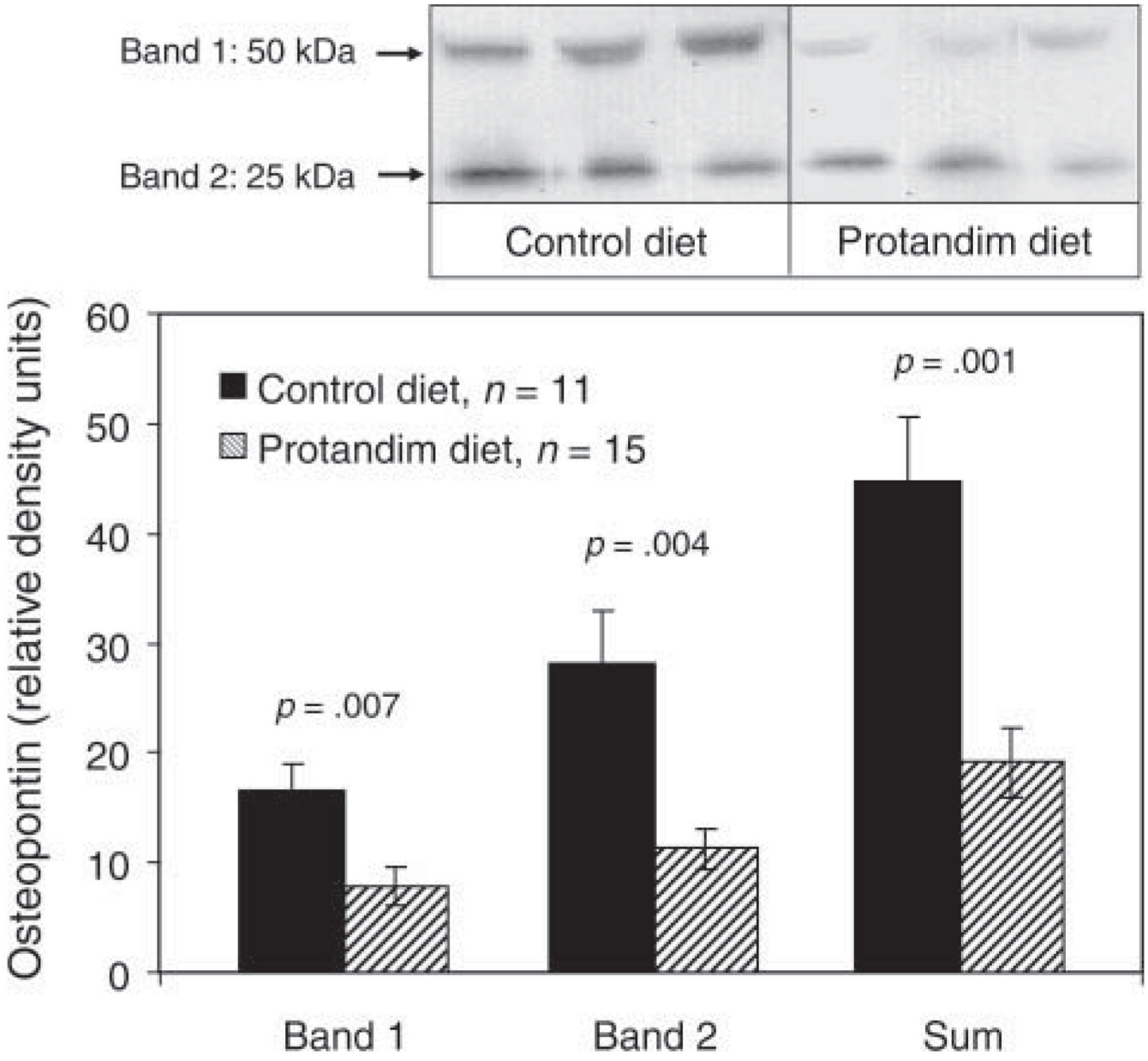

FIGURE 3.

(a) Western blot analysis for OPN in 6 month mdx mice, showing three representative animals from each group with total densities near the mean for their group. (b) The mean integrated relative densities ± SEM for the two groups are shown for each band, and for the sum of the two bands for each animal. An internal standard was used to normalize multiple blots.

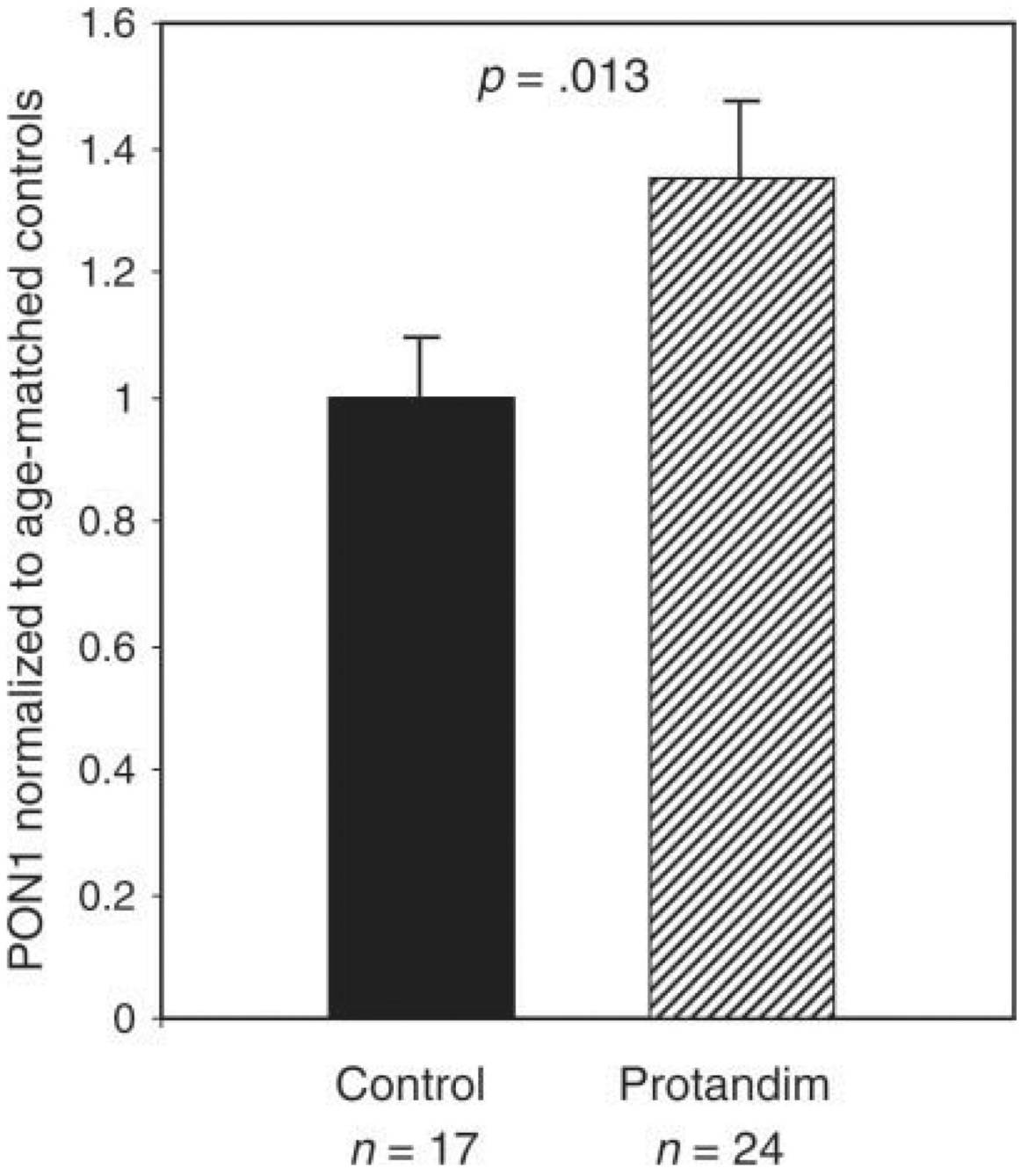

FIGURE 4.

Protandim® increases plasma PON1 normalized to the mean for age-matched control diet animals. At 6 weeks, Protandim®-supplemented animals averaged 30.1 ± 2.6 U/ml or 35% more than control diet group (22.3 ± 3.1 U/ml). Similarly, at 6 months, Protandim-supplemented animals averaged 11.7 ± 1.4 U/ml or 36% more than control diet group (8.6 ± 1.1 U/ml), even though plasma PON1 activity dropped for both groups from 6 weeks to 6 months.

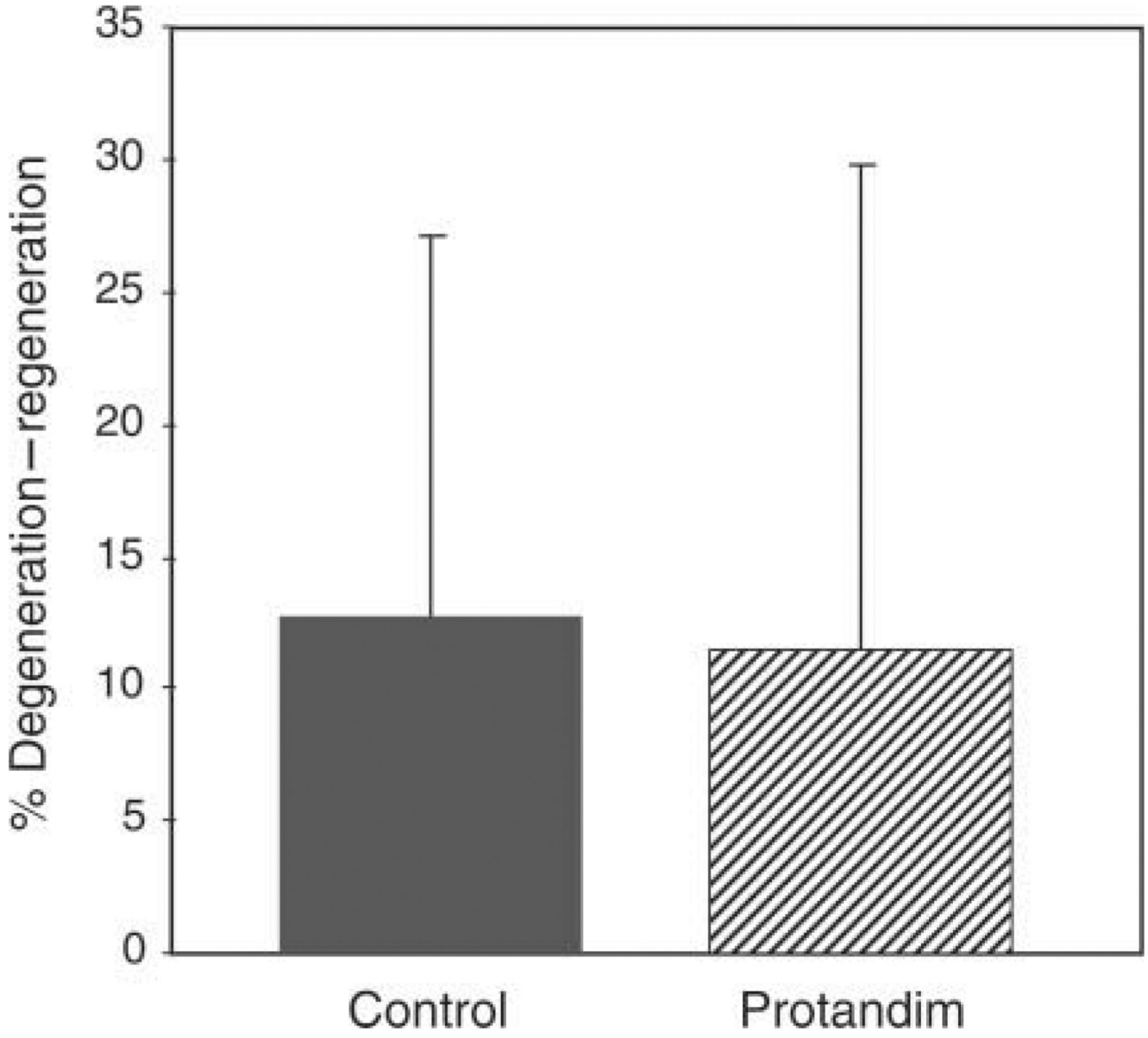

FIGURE 5.
H&E comparisons in the quadriceps muscles of Protandim® versus control rodent diet fed in 6-week-old *mdx* mice demonstrate increased percentage degeneration–regeneration in the control group (however statistically not significant).

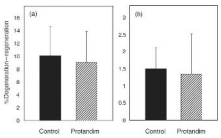

FIGURE 6.

H&E comparisons in Protandim® versus control rodent diet fed in 6-month-old *mdx* mice demonstrate increased % degeneration–regeneration in the diet control group (statistically not significant) in the (a) gastrocnemius muscle (b) rectus femoris muscle.

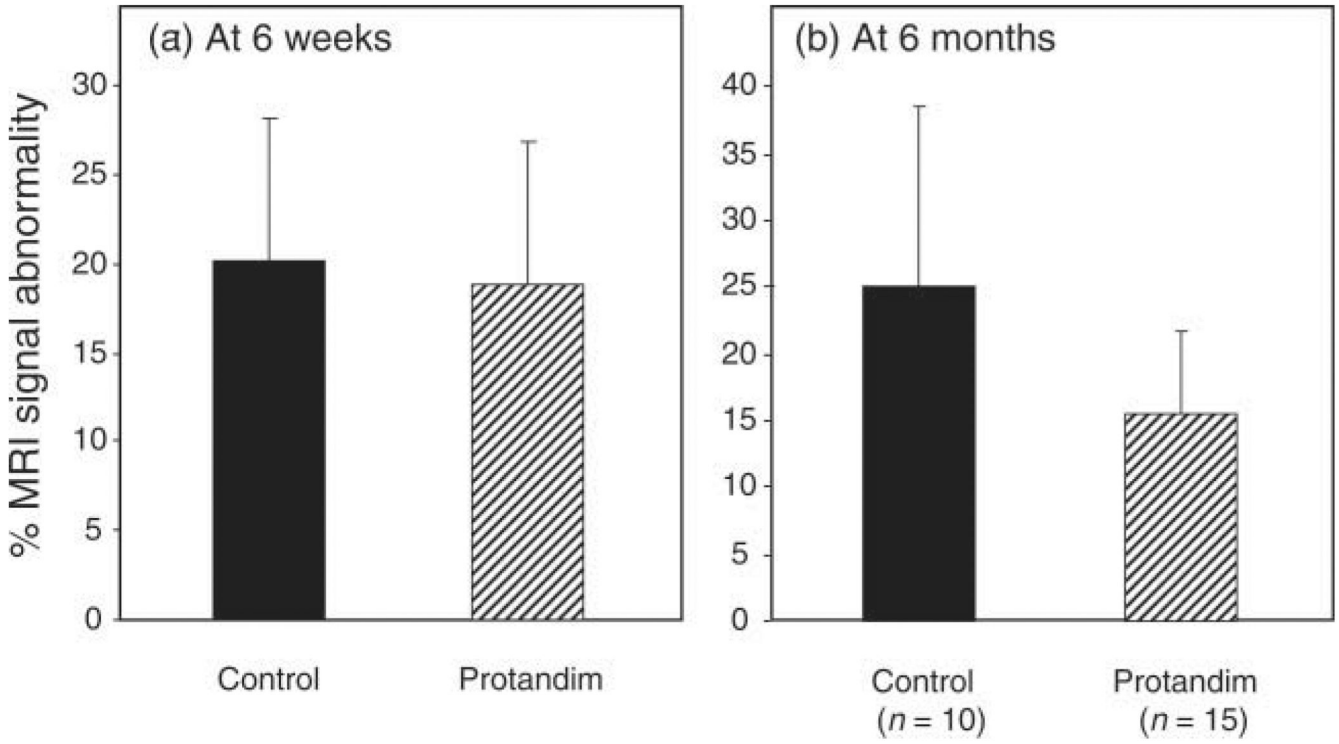

FIGURE 7.

ROI-quantitative measurement of percentage gadolinium-enhanced MRI areas in the control-fed group (25.1% ± 13.6% SD, $n = 10$) compared with the Protandim®-fed group (15.6% ± 6.2% SD, $n = 15$) at 6 months showed a mean 38% reduction in the Protandim group, but did not reach statistical significance ($p = .07$).

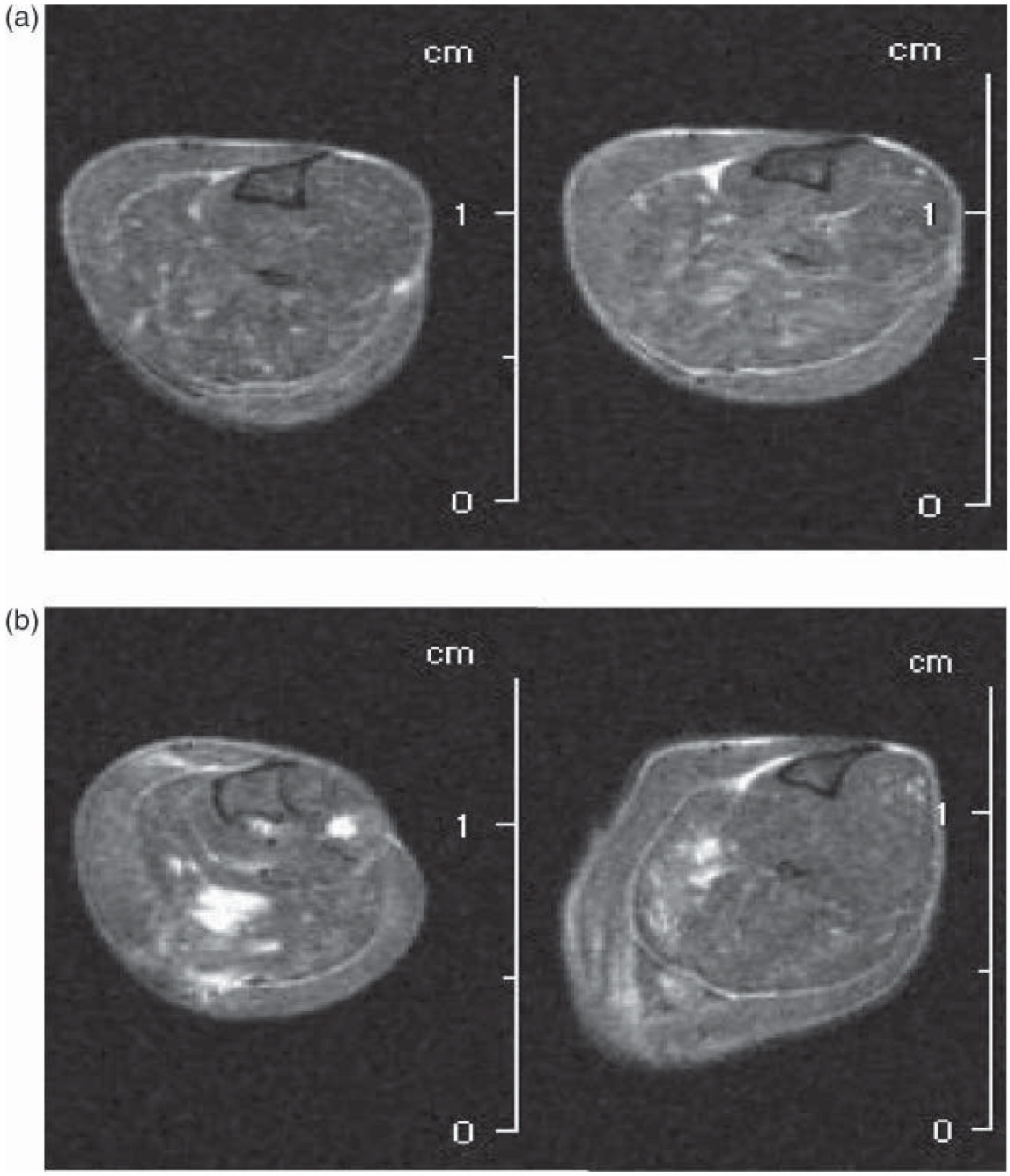

FIGURE 8.

Representative MRI muscle images in the lower leg of 2 Protandim®-diet and 2 control-diet fed *mdx* mice at 6 months. The brighter areas reflected distinct MRI signal contrast enhancement: black: bone cavities; white: fat and conjunctive tissues; grey:muscles. (a) No enhanced white signal is visible on cross-sections of Protandim® -fed mice, although subcutaneous fat (nonmuscle region) gives similar signal. (b) More distinct gadolinium-enhancing (white) regions are visible on the muscles on cross-sections of the control diet in *mdx* mice. Gadolinium-enhancing muscle regions were quantified and there was a 35% decrease after 6 months of Protandim® diet ($p = .07$).

TABLE 1

Morbidity and Mortality in Male Mdx Mice Supplemented with Protandim® or Control Diet

	Duration	*n*	Age (Pretreatment) Days	Age (Posttreatment) Days	Deaths (Pre-MRI)	Deaths (Post-MRI)
Protandim diet	6 weeks	13	*in utero*	42	1	0
	6 months	18	42	182	0	2
Control diet	6 weeks	12	*in utero*	42	0	1
	6 months	12	42	182	0	1

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